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Pathology

THIRD EDITION

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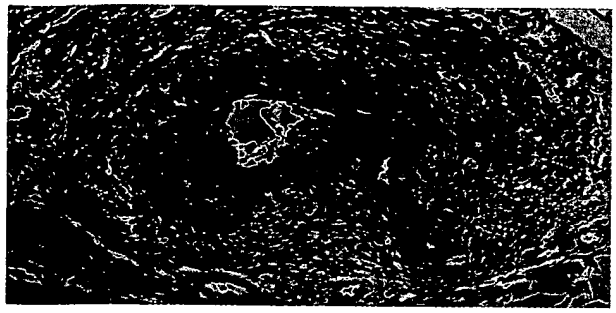

FIGURE 1-22
Fibrinoid necrosis. An inflamed muscular artery in a case of systemic arteritis shows a sharply demarcated, homogeneous, deeply eosinophilic zone of necrosis.

of coagulative necrosis, can account for the common morphology of cell death. The sequence of events leading to coagulative necrosis may then be described as (1) irreversible injury and cell death, (2) loss of the plasma membrane's ability to maintain a gradient of calcium ions, (3) an influx and accumulation of calcium ions in the cell, and (4) the morphological appearance of coagulative necrosis. Under such a scheme, coagulative necrosis occurs after the point of no return—that is, after irreversible injury and "death" of the cell.

Alternatively, cell injury may lead to potentially reversible plasma membrane damage. In such a scheme, the large gradient of calcium ions can no longer be maintained by the plasma membrane. Excess calcium ions then accumulate in the injured cells and cause coagulative necrosis. This scenario has two specific implications. First, it does not define a stage of cell death distinct from coagulative necrosis. Second, it envisions the accumulation of calcium ions as the point at which potentially reversible cell injury becomes irreversible. There are some experimental data to support such a hypothesis. Agents that block calcium fluxes across biological membranes have been shown to prevent the coagulative necrosis that usually follows reperfusion of liver cells otherwise irreversibly injured by ischemia. Such experiments are difficult to interpret, however, because we cannot specifically implicate the inhibition of calcium accumulation as the mechanism by which the drug prevents coagulative necrosis. Any alternative action of the drug that results in cytoprotection would similarly prevent the accompanying accumulation of calcium.

The preceding discussion is summarized as follows: **Whatever the role of calcium, the disruption of the permeability barrier of the plasma membrane seems to be a critical event in lethal cell injury.** Loss of the plasma membrane's barrier function results in an equilibration of the concentration gradients that characterize living cells. As these gradients are dissipated, the cells are transformed into necrotic debris.

Ischemic Cell Injury

The interrupti n of blood flow—ischemia—is probably the most important cause of c agulative necrosis in human disease. The complications of atherosclerosis, for example, are generally the result of ischemic cell injury in the brain, heart, small intestine, kidneys, and lower extremities. Highly differentiated cells, such as the proximal tubular cells of the kidney, cardiac myocytes, and the neurons of the central nervous system, depend on aerobic respiration to produce ATP for the performance of their specialized functions. When ischemia limits the supply of oxygen and ATP is depleted, these cells rapidly manifest many changes in structure and function.

The effects of ischemic injury are all reversible if the duration of ischemia is short. For example, changes in myocardial contractility, membrane potential, metabolism, and ultrastructure are short lived if the circulation is rapidly restored. However, when ischemia persists, the affected cells become irreversibly injured—that is, the cells continue to deteriorate and become necrotic despite reperfusion with arterial blood. By definition, all metabolic alterations associated with reversible ischemic cell injury are either quantitatively or qualitatively insufficient to produce irreversible injury. With longer periods of ischemia, some biochemical alteration develops that causes irreversible injury.

Two phenomena illustrate the difference between irreversibly and reversibly injured ischemic cells. An inability to reverse mitochondrial dysfunction on reperfusion or reoxygenation correlates with a similar inability to reverse the cell injury in general. This finding was originally interpreted as indicating that ischemic cell death is a consequence of irreversible mitochondrial injury, particularly since mitochondria develop a series of structural and functional abnormalities with ischemia. However, it has been shown that the environment to which reperfusion exposes irreversibly injured cells does not allow mitochondria to recover from injury that would otherwise be reversible. In particular, it has been demonstrated that during reperfusion of irreversibly injured cells a large influx of Ca^{2+} ions occurs. An excess of Ca^{2+} ions is known to induce loss of mitochondrial function, and it may be that the inability to reverse mitochondrial dysfunction reflects the flooding of the cells with Ca^{2+}, rather than being a consequence of the mitochondrial abnormalities themselves.

Recent evidence has renewed interest in mitochondrial injury as a key element in the pathogenesis of irreversible cell injury. Under ischemic conditions, mitochondria exhibit a nonspecific increase in the permeability of the inner membrane. This effect is attributed to the opening of a proteinaceous pore in the membrane (mitochondrial permeability transition). Agents that inhibit this transition protect against irreversible ischemic cell injury, both *in vitro* and in the intact animal. Thus, mitochondria are now again identified as potentially critical in the development of ischemic cell death.

A disturbance in membrane function in general, and in the plasma membrane in particular, is the second characteristic of the loss of reversibility in ischemic injury. Some have therefore maintained that defective cell membrane function is the primary event in the genesis of irreversible cell injury in ischemia. Indeed, the results of morphological, functional, and biochemical studies clearly suggest that defects in cell membranes are an early feature of irreversible ischemic cell injury. Yet a definitive understanding of the mechanism underlying membrane dam-

age in irreversible ischemic injury remains elusive. There are, however, potential candidates for this mechanism.

Reperfusion Injury and Activated Oxygen

A popular theory postulates a role for partially reduced, and thus activated, oxygen species in the genesis of membrane damage in irreversible ischemia. The general problem of how activated oxygen species may injure cells is discussed later in this chapter; here, we consider the mechanisms by which activated oxygen (superoxide, H_2O_2, hydroxyl radicals) is formed in ischemia and the evidence that it injures ischemic cells.

It might seem paradoxical that oxygen species cause cell injury when that injury is attributed to an insufficient oxygen supply. This dilemma is more apparent than real. Toxic oxygen species are generated not during the period of ischemia itself but rather on restoration of blood flow, or reperfusion, hence the term **reperfusion injury**.

Some event occurs during the period of ischemia that results in an overproduction of toxic oxygen species on the later restoration of the oxygen supply. Two sources of activated oxygen species have been proposed, namely, production by intracellular xanthine oxidase and extracellular release by activated neutrophils.

In some circumstances, for example as originally described with experimental intestinal ischemia, xanthine dehydrogenase may be converted by proteolysis during the period of ischemia into xanthine oxidase. On return of the oxygen supply with reperfusion, the abundant purines derived from the catabolism of ATP during ischemia provide substrates for the activity of xanthine oxidase. This enzyme requires oxygen in catalyzing the formation of uric acid, and activated oxygen species are byproducts of this reaction.

A second source of activated oxygen species during reperfusion may be the neutrophil. It is thought that alterations in the cell surface that occur during ischemia and on reperfusion induce the adhesion and activation of circulating neutrophils. These cells release large quantities of activated oxygen species and hydrolytic enzymes, both of which may injure the previously ischemic cells. This concept is supported by the protection afforded by reperfusing ischemic tissue with blood depleted of neutrophils. In addition to the generation of toxic oxygen species in reperfusion injury, other agents, such as cytokines, nitric oxide, platelet activating factor (PAF), and other molecules, have been proposed as mediators or modulators of tissue damage.

The specific role that reperfusion injury plays in the genesis of irreversible ischemic injury in human disease remains to be defined. We can put reperfusion injury in perspective by emphasizing that there are three different degrees of cell injury, depending on the duration of the ischemia:

1. With short periods of ischemia, reperfusion (and, therefore, the resupply of oxygen) completely restores the structural and functional integrity of the cell. Cell injury in this case is completely reversible.
2. With longer periods of ischemia, reperfusion is not associated with restoration of cell structure and function but rather with deterioration and death of the cells. As we have seen, this seemingly paradoxical response to reoxygenation is a consequence of the formation of reduced oxygen species on reperfusion, and it is these activated oxygen species that injure the cells. It is important to emphasize that, in this case, lethal cell injury occurs during the period of reperfusion.
3. Lethal cell injury may develop during the period of ischemia itself, in which case reperfusion is not a factor. A longer period of ischemia is needed to produce this third type of cell injury. In this case, cell damage is not dependent on the formation of activated oxygen species. When cells are reperfused after periods of ischemia that produce this type of injury, there is an explosive accumulation of sodium and calcium ions in the cells. This accumulation is a result of plasma membrane damage that developed during the period of ischemia—not during reperfusion.

The damage to the plasma membrane directly associated with irreversible ischemic injury, and not dependent on reperfusion, has been attributed to two mechanisms. One of these mechanisms relates to changes in the metabolism of the phospholipid bilayer, whereas the other emphasizes alterations in cytoskeletal structures.

Altered Phospholipid Metabolism

Plasma membrane damage in irreversible ischemia has been attributed, at least in part, to accelerated degradation of membrane phospholipids. Evidence for this hypothesis has been derived in a number of experimental systems, including liver, heart, brain, and kidney ischemia.

Experimental ischemia has been shown to result in a loss of phospholipids from cell membranes, accompanied by a release of their fatty acids. Structural alterations in cellular membranes accompany this increased hydrolysis of phospholipids. Microsomes and plasma membranes display aggregations of intramembranous particles, a finding suggestive of phase separations in the lipid bilayer. Interfaces between lipid domains of differing molecular order are believed to be sites of increased permeability. Indeed, microsomal membranes prepared from ischemic livers exhibit a 25- to 50-fold increase in their passive permeability to calcium.

The mechanism responsible for the accelerated degradation of membrane phospholipids in ischemia is not fully understood. It has been proposed that membrane-associated phospholipases are activated by increases in cytosolic free calcium during ischemia. According to this hypothesis, energy depletion dissipates the mitochondrial gradient that permits the retention of calcium in that organelle. With ischemia, the release of mitochondrial calcium elevates the cytosolic calcium concentration and activates phospholipases. Support for this hypothesis comes from studies of cardiac myocytes, in which cytosolic calcium has been reported to increase at the same time that fatty acids are released from the phospholipids of cellular membranes. By contrast, phospholipid degradation in anoxic liver cells can proceed in the

absence of an elevated cytosolic free calcium. Thus, the role of phospholipase activation by calcium in ischemic injury remains to be defined.

Cytoskeletal Alterations

The intimate association of cytoskeletal elements with the plasma membrane of many cells suggests that the cytoskeleton plays a role in the regulation of the structure of the cell membrane. Thus, it has been postulated that alterations in the cytoskeleton provide another mechanism by which ischemia damages the plasma membrane. Evidence has been forthcoming that anoxia may lead to activation of phospholipases by interfering with the intimate relationship between the cytoskeleton and the plasma membrane. In a number of experimental systems, both ischemia and anoxia lead to the formation of prominent blebs of the plasma membrane, which have been attributed to cytoskeletal changes. These fluid-containing blebs are not seen in reversibly injured cells. The biochemical basis for the formation of plasma membrane blebs, or for the postulated participation of the cytoskeleton, remains to be elucidated.

It is of interest that although the participation of the cytoskeleton in ischemic injury is not entirely defined, direct modification of the cytoskeleton can have profound effects on the viability of the cell. The best example is the liver injury produced by phalloidin, one of the active agents of the toxic mushroom *Amanita phalloides*. Phalloidin binds to actin filaments, thereby preventing their depolymerization. The resulting accumulation of microfilaments in immediate association with the plasma membrane produces numerous invaginations of that structure

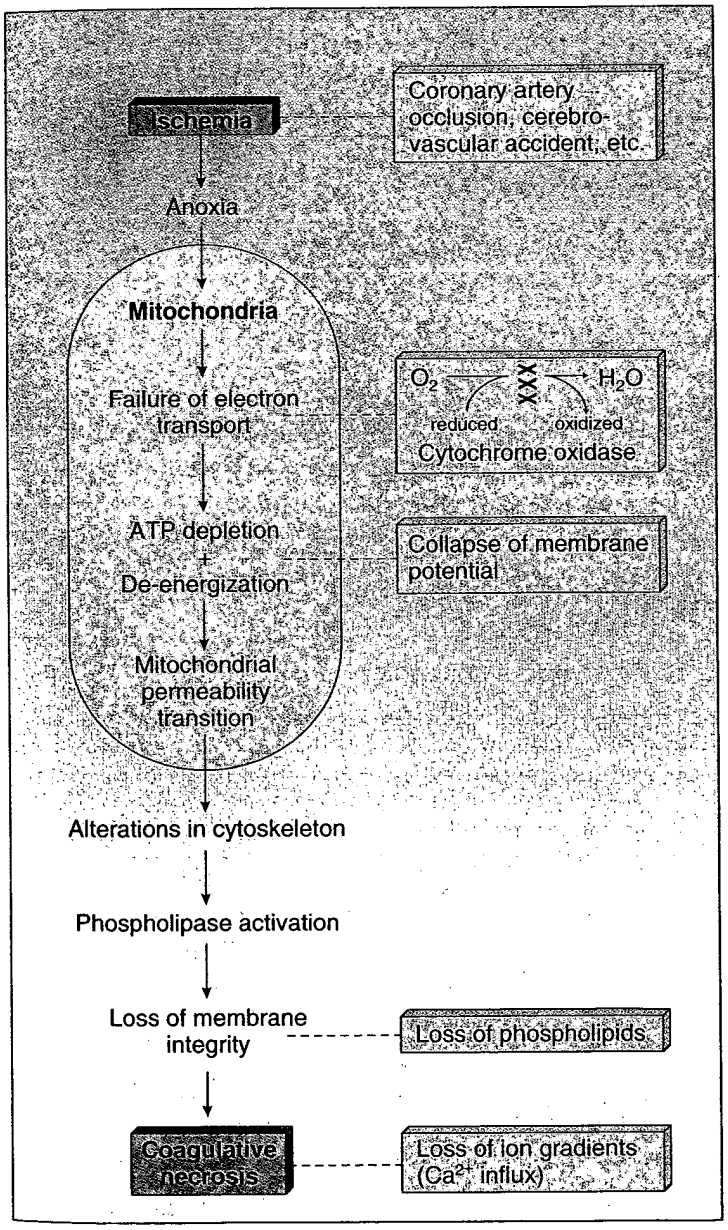

FIGURE *1-23*
Possible sequence of events in the pathogenesis of irreversible cell injury caused by anoxia/ischemia.

and eventually lethal injury to the cell. The fungal metabolite cytochalasin B enhances the depolymerization of actin and prevents the toxicity of phalloidin.

The events leading to irreversible ischemic cell injury are summarized in Figure 1-23.

Cell Injury Caused by Oxygen Free Radicals

Partially reduced oxygen species have been identified as the likely cause of cell injury in an increasing number of diseases (Fig. 1-24). We referred earlier to reperfusion injury when discussing the mechanism of cell injury in ischemia. The inflammatory process, whether acute or chronic, can cause considerable tissue destruction. Partially reduced oxygen species produced by phagocytic cells are important mediators of cell injury in such circumstances. Damage to cells resulting from oxygen radicals formed by inflammatory cells has been implicated in diseases of the joints and of many organs, including the kidney, lungs, and heart. The toxicity of many chemicals may reflect the formation of toxic oxygen species. The killing of cells by ionizing radiation is most likely the result of the direct formation of hydroxyl radicals from the radiolysis of water. There is also evidence of a role for oxygen species in chemical carcinogenesis, during eit initiation or promotion. Finally, oxidative damage been implicated in biological aging (see later).

Cells also may be injured when oxygen is presen concentrations greater than normal. In the past, this curred largely in those therapeutic circumstances which oxygen was given to patients at concentrati greater than the normal 20% of inspired air. The lung adults and the eyes of premature newborns were the jor targets of such oxygen toxicity.

Oxygen has a major metabolic role as the terminal ceptor for mitochondrial electron transport. Cytochrc oxidase catalyzes the four-electron reduction of O_2 to ter. The resultant energy is harnessed as an electroche cal potential across the mitochondrial inner membran

There are three partially reduced species that are termediate between O_2 and H_2O, representing transfer varying numbers of electrons. They are O_2^-, superox (one electron); H_2O_2, hydrogen peroxide (two electro and ·OH, the hydroxyl radical (three electrons). Th partially reduced oxygen species are not produced by tochrome oxidase but are derived from other enzym and nonenzymatic reactions (Fig. 1-25).

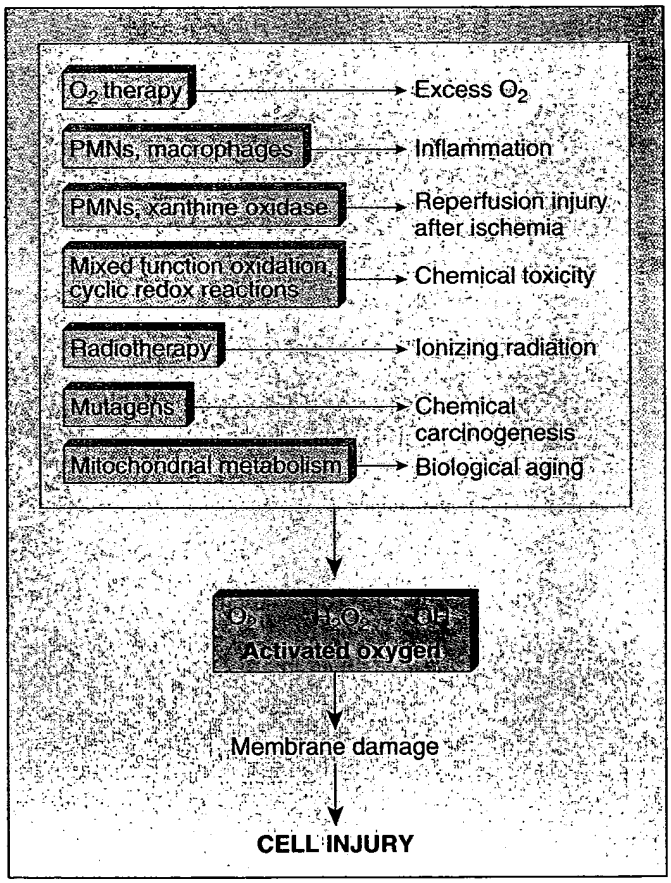

FIGURE *1-24*
The role of activated oxygen species in human disease.

Superoxide

Components of the mitochondrial electron transp chain may be directly auto-oxidized by O_2 to yield peroxide anions (O_2^-). Superoxide anions are also p duced by enzymes such as xanthine oxidase and tochrome P_{450}. Phagocytosis by polymorphonuc leukocytes and macrophages is accompanied by creased oxygen consumption, which largely repres the formation of O_2^- by an oxidase in the plasma m brane. O_2^- anions produced in the cytosol or mitoch dria are catabolized by superoxide dismutase. molecule of H_2O_2 and one molecule of O_2 are forn from two molecules of O_2^-. Hydrogen peroxide is produced directly by a number of oxidases in cytop mic peroxisomes (see Fig. 1-25).

Hydrogen Peroxide

Most cells have efficient mechanisms for removing H Two different enzymes reduce H_2O_2 to water: cata within the peroxisomes and glutathione peroxidas both the cytosol and the mitochondria (see Fig. 1-25). tathione peroxidase uses reduced glutathione (GSH) co-factor, producing two molecules of oxidized tathione (GSSG) for every molecule of H_2O_2 reduce water. GSSG is re-reduced to GSH by glutathione re tase, with reduced nicotinamide adenine dinucleo phosphate (NADPH) as the co-factor.

Hydroxyl Radical

Hydroxyl radicals are known to be formed in biolog systems in only two ways: by the radiolysis of water o the reaction of hydrogen peroxide with ferrous iron Fenton reaction).

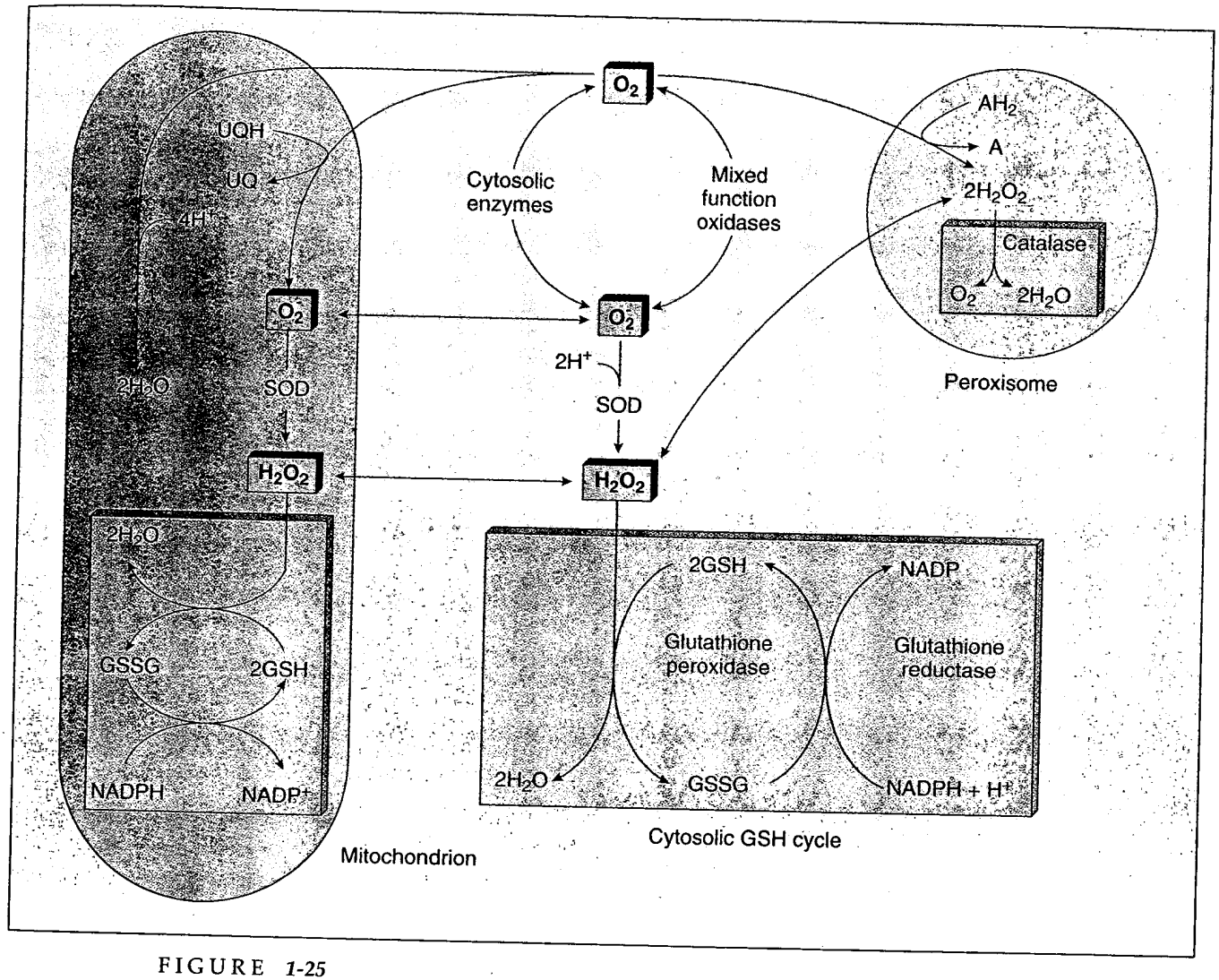

FIGURE *1-25*
Cellular metabolism of oxygen and the accompanying antioxidant defense mechanisms.

The Role of Iron

All respiring cells require iron; it is used, for example, to form cytochromes for electron transport in the mitochondria. Cells obtain iron from the plasma as ferric iron bound to transferrin. Transferrin binds to specific receptors on the cell surface and is delivered to the cytoplasm within an endosome, where an acidic environment releases free ferric iron. Free iron first is used for the synthesis of hemoproteins and then is stored as ferritin; it is subsequently returned to newly synthesized or recycled transferrin and is then secreted by the cell. Cellular iron stores may be mobilized by the autophagocytosis of ferritin. Following the fusion of autophagosomes with lysosomes, the acid proteases activated by the low pH release free ferric iron. Figure 1-26 summarizes these events, emphasizing the presence of a **pool of free ferric iron f rmed as a result f both the uptake and the release f iron fr m cells.**

It is this pool of free ferric iron that seems to be required for partially reduced oxygen species to injure cells. Free ferric iron can be reduced by superoxide anions to ferrous iron. Hydrogen peroxide, formed either directly or (more commonly) by the dismutation of superoxide anions, then reacts with the ferrous iron by the Fenton reaction to produce hydroxyl radicals. This sequence, starting with superoxide anions and ferric iron and leading to the generation of hydroxyl radicals without the consumption of ferric iron, is called an iron-catalyzed Haber-Weiss reaction.

Hydroxyl Radicals and Macromolecules

The hydroxyl radical (·OH) is an extremely reactive species, and there are several mechanisms by which it might damage membranes.

- **Lipid peroxidation:** The best known effect of hydroxyl radicals on membranes relates to ·OH as an initiator of lipid peroxidation (Fig. 1-27). The hy-

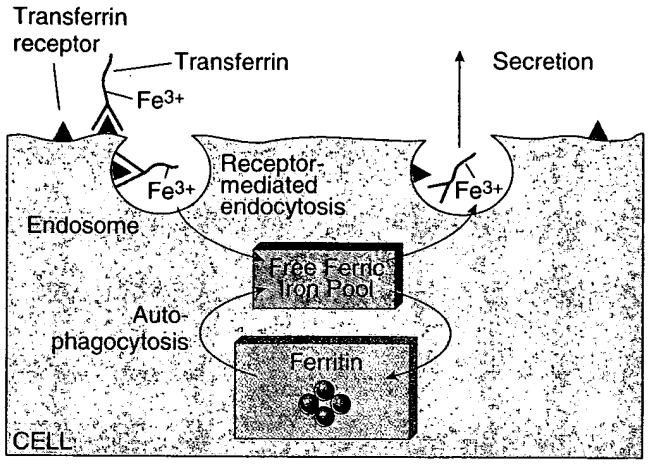

FIGURE *1-26*
Cellular metabolism of iron.

droxyl radical removes a hydrogen atom from the unsaturated fatty acids of membrane phospholipids, a process that forms a free lipid radical. The lipid radical, in turn, reacts with molecular oxygen and forms a lipid peroxide radical. Like ·OH, this peroxide radical can function as an initiator, removing another hydrogen atom from a second unsaturated fatty acid. A lipid peroxide and a new lipid radical result, and a chain reaction is initiated.

Lipid peroxides are unstable and break down into smaller molecules (hydroxyaldehydes) that either remain attached to the glycerol backbone of the phospholipid or are released into the cytosol. The destruction of the unsaturated fatty acids of phospholipic results in a loss of membrane integrity. Antioxidant such as vitamin E, prevent the injury that usually fo lows exposure of cells to partially reduced oxyge species. This protection is attributed to the inhibitio of lipid peroxidation by antioxidants.

- **Protein interactions:** Hydroxyl radicals may als damage membranes by altering their proteins. The may cause cross-linking of membrane protein through the formation of disulfide (S-S) bonds. Th resulting aggregation of membrane proteins ma form ion channels or may otherwise disrupt mem brane structure and function. The SH groups of mem brane proteins can also be modified by the formatio of mixed disulfides in a reaction with GSH, a proces dependent on the hydroxyl radical. The products c lipid peroxidation form carbonyl adducts with intra cellular proteins, thereby providing a convenier marker of oxidative damage. The modification c membrane proteins has been suggested as an alterna tive to lipid peroxidation as a mechanism by whic oxygen species produce irreversible cell injury, al though the two are not necessarily exclusive.
- **DNA damage:** DNA is an important target of the hy droxyl radical. A variety of structural alterations ir clude strand breaks, modified bases, and cross-link between strands. In most cases, the integrity of th genome can be reconstituted by the various DNA re pair pathways. However, if oxidative damage t DNA is sufficiently extensive, the cell dies.

Figure 1-28 summarizes the mechanisms of cell injur by activated oxygen species.

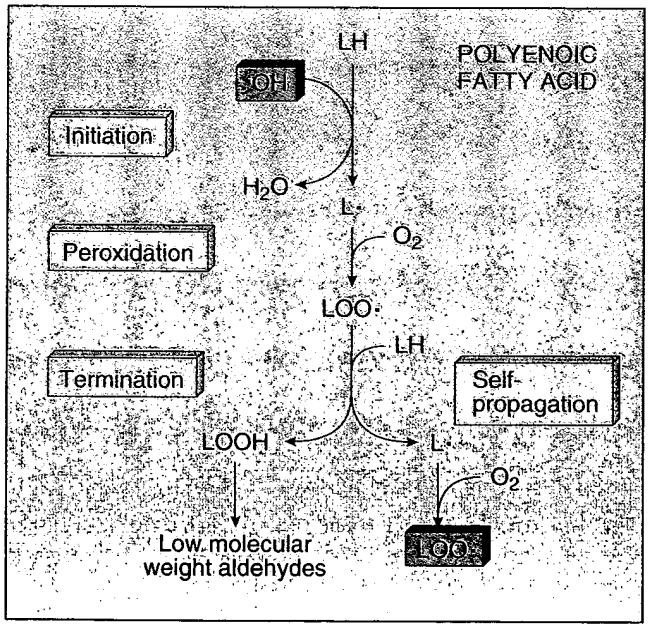

FIGURE *1-27*
Lipid peroxidation initiated by the hydroxyl radical.

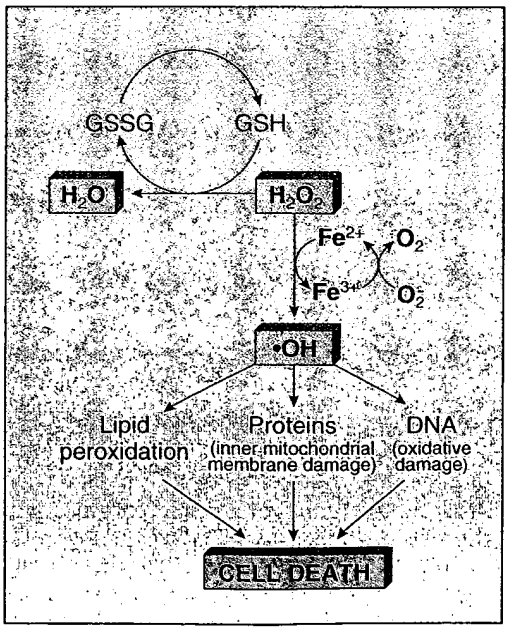

FIGURE *1-28*
The mechanisms of cell injury by activated oxygen species